PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48 A2

(11) International Publication Number: WO 96/20012

(43) International Publication Date: 4 July 1996 (04.07.96)

(21) International Application Number: PCT/US95/16950

(22) International Filing Date: 22 December 1995 (22.12.95)

(30) Priority Data:
08/372,820 23 December 1994 (23.12.94) US
08/538,817 4 October 1995 (04.10.95) US

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(71) Applicant: MIDDLESEX SCIENCES, INC. [US/US]; 220 Norwood Park South, Norwood, MA 02062 (US).

(72) Inventors: DI, Jie; 350 Engamore Lane #208, Norwood, MA 02062 (US). WOISZWILLO, James, E.; 3 Tara Circle, Milford, MA 01757 (US).

(74) Agents: GREENE, Jamie, L. et al.; Jones & Askew, 37th floor, 191 Peachtree Street, N.E., Atlanta, GA 30303-1769 (US).

(54) Title: METHODS FOR PREPARING AND PURIFYING MACROMOLECULAR CONJUGATES

(57) Abstract

Methods for the preparation and purification of conjugates by reacting a molecule containing an aldehyde group with a molecule containing an amine group in the presence of a polymer for a sufficient amount of time to form conjugate particles. Conjugates are prepared rapidly and efficiently by oxidizing the aldehyde and combining the oxidized aldehyde with a protein and polymer solution to form Schiff base intermediate conjugate particles in the absence of reductive amination. Subsequent separation of the particles from the conjugation reaction mixture yields purified conjugates that have been separated from the free, unconjugated components. Most preferably, the polymer is a polymer mixture comprising polyvinylpyrrolidone and polyethylene glycol. An organic solvent, such as an alcohol may be added to the incubation mixture to facilitate particle formation. A conjugating agent may also be included in the reaction mixture. The particles may be washed for removal of additional undesired contaminants and solubilized with a base, yielding soluble conjugates. Conjugates prepared from one or more antigens are suitable for use as a vaccine when administered to humans or animals.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia
AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CS	Czechoslovakia
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgystan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakhstan
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam

METHODS FOR PREPARING AND PURIFYING MACROMOLECULAR CONJUGATES

Field of the Invention

This relates to the field of biochemistry and more particularly relates to the preparation and purification of macromolecular conjugates.

Background of the Invention

Infants are routinely given a series of vaccinations within the first few months of life to provide protection against potentially life-threatening bacterial and viral diseases. Some antigens, especially the carbohydrate antigens of bacteria, are either poorly immunogenic or not immunogenic in children younger than 18 to 24 months of age. To date, all conjugate vaccines are generated by the chemical linkage of carbohydrate to protein. For example, an antigen such as the capsular polysaccharide of *Haemophilus influenzae* type b (Hib), one of the major causative organisms of bacterial meningitis, is conjugated to a carrier protein for successful induction of an antibody response in infants.

Approximately 12,000 new cases of bacterial meningitis are reported yearly, primarily in children under five years of age. Despite antimicrobial therapy, the mortality rate from meningitis is 3-8%. Neurologic sequelae are observed in as many as 25% to 35% of the survivors, at an estimated cost of more than half a million dollars over the lifetime of those children. *Haemophilus influenzae* type b is the etiologic agent for other diseases such as epiglottitis, sepsis, cellulitis, septic arthritis, osteomyelitis, pericarditis, and pneumonia (*MMWR* 34:201-205 (1985)). Studies have indicated that the risk for children under five years of age of acquiring a *Haemophilus influenzae* type b disease appears to be

greater for those enrolled in daycare facilities (Istre, G.R., et al., *J. Pediatr.* 106:190-195 (1985) and Redmond, S.R., et al., *J. Am. Med. Assoc.* 252:2581-2584 (1984)).

Two types of lymphocytes, B lymphocytes and T lymphocytes, are normally involved in the development of immunity. Some antigens (T-independent antigens) are capable of stimulating B cells to produce antibody in the absence of T lymphocyte involvement. However, most antigens (T-dependent antigens) require the participation of both B lymphocytes and T lymphocytes to produce an effective immune response, a process often referred to as cell mediated immunity, in which antigen-presenting cells present processed antigen to helper T cells, which secrete lymphokines that promote the production of antibodies by B cells and influence other cells of the immune system. Young infants, less than 18 months old, fail to respond to T-independent antigens for reasons that are not yet fully understood. Therefore, a T-dependent antigen is preferred for use as a vaccine because it results in an enhanced antibody response, the production of immunoglobulins of all classes, and the development of immunologic memory. The covalent binding of antigens has been used to convert T-independent antigens into T-dependent antigens, thereby creating more effective vaccines.

A variety of chemical linkages have been used to prepare antigen conjugates. However, the coupling methods currently employed are time-consuming, thereby increasing the costs of production. The chemical reactions employed are also difficult to control, often resulting in linkages such as amido linkages that cause excessive crosslinking or detrimental modifications to the antigen. These are undesirable characteristics in human vaccines. The coupling chemistries currently in use provide a low yield of conjugate, thereby wasting expensive antigen preparations and requiring extensive purification, adding to the costs of production. For example, polysaccharides may be coupled to proteins using reductive amination as described in U.S. Patent No. 4,356,170 to Jennings et al., entitled "Immunogenic Polysaccharide-Protein Conjugates". Conjugation is achieved by oxidizing the polysaccharide with an oxidizing agent, coupling the oxidized polysaccharide to a protein, and reducing the bond with a reducing agent for stability. This process requires lengthy dialysis

and incubation steps and chromatography purification and may take up to two to three weeks for completion.

Purification of carbohydrate-protein conjugates to remove non-reacted components and conjugation reagents contained in the conjugation reaction mixture is essential. Current purification strategies include size exclusion column chromatography, ion exchange column chromatography, and ammonium sulfate precipitation. These techniques are time-consuming and expensive. Furthermore, some carbohydrate-protein conjugates are insufficiently purified using these methods.

In addition to the conjugation of carbohydrates to proteins, scientists frequently conjugate proteins to other molecules such as other proteins, peptides, catecholamines, transmitters, hormones, receptors, nucleic acids, or chemicals for diagnostic, therapeutic, and research purposes. For example, a small, non-immunogenic peptide may be conjugated to a large, carrier protein, such as bovine serum albumin, to elicit an immune response to the small, non-immunogenic protein when the conjugate is administered to a human or animal. In addition, a small detectable protein such as an enzyme may be coupled by conjugation to a protein, such as an antibody or receptor, for detection of a ligand by an enzymatic reaction that produces a detectable signal. Furthermore, a non-protein chemical such as a fluorescent or radioactive compound or biotin may be coupled to a protein for use as a label to detect a ligand to which the protein has an affinity. Current methods for the purification of these conjugates involve long periods of dialysis or other types of lengthy and cumbersome separation techniques. These conjugates are commonly used quantitatively. If the conjugate is improperly purified, the residual non-conjugated protein may compromise quantitative experimental or diagnostic results or undermine therapeutic effects. Therefore, methods for the purification of conjugates from the unconjugated reagents, such as unconjugated protein, are essential.

What is needed are rapid, efficient and inexpensive conjugation preparation and purification methods that results in stable, purified macromolecular conjugates.

Summary of the Invention

Methods are provided for preparing and purifying macromolecular conjugates through the formation of conjugate particles. The methods are particularly useful for the preparation and purification of carbohydrate-protein conjugates from chemical reaction mixtures for administration as vaccines to young humans or animals.

Conjugate particles are formed in a first preferred embodiment by reacting a reaction mixture containing a molecule having a reactive amine group, a molecule having a reactive aldehyde group, and a polymer or mixture of polymers for a sufficient amount of time to form conjugate particles. Preferably, the mixture is incubated at temperatures between approximately 20° C and 65° C for a period of time between approximately 15 minutes and 5 hours at a pH near the pI of the resulting conjugate. In a second preferred embodiment, the components to be conjugated may be any molecules capable of being conjugated, and the reaction mixture further includes a conjugating agent. In a third preferred embodiment, pre-formed conjugates are reacted with the polymer to produce conjugate particles. An organic solvent may be added to any of the foregoing reaction mixtures to facilitate particle formation. The preferred organic solvent is an alcohol. The latter two embodiments are useful for purification of conjugates prepared by known conjugation reactions.

In the first preferred embodiment, the molecule having a reactive aldehyde group is one that either contains a reactive aldehyde group or can be modified to expose or otherwise include a reactive aldehyde group. The reactive aldehyde group is one having the ability to react with and couple to an amine group. Such a molecule includes, but is not limited to, a carbohydrate, glycoprotein, peptide, protein, catecholamine, transmitter, hormone, receptor, label, nucleic acid or chemical. The carbohydrate includes, but is not limited to, lipopolysaccharides, polysaccharides, and oligosaccharides derived from microorganisms such as bacteria, fungi, protozal groups, and virally associated lipopolysaccharides, polysaccharides and oligosaccharides. The molecule having a reactive amine group is a molecule having a tertiary structure, preferably a protein or peptide, and includes, but is not limited to, toxoids; toxins; exotoxins; proteins derived from animals, plants, viruses, bacteria, fungi, and protozoa; recombinant proteins; and synthetic proteins. The reactive amine

group is one having the ability to react with and couple to an aldehyde group.

The polymers used to form the conjugate particles may be water soluble, semi-water soluble, or non-water soluble and can include the following categories of polymers: carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers and block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched and cyclo-polymers, and polyaldehydes, and mixtures thereof. The polymer is preferably polyvinylpyrrolidone, polyethylene glycol, dextran, polyoxyethylene-polyoxypropylene copolymer, polyvinyl alcohol, or mixtures thereof. Most preferably, the polymer is a polymer mixture comprising polyvinylpyrrolidone and polyethylene glycol. The preferred molecular weight of the polyvinylpyrrolidone is approximately 40,000; the preferred molecular weight of the polyethylene glycol is approximately 3350; the preferred concentration of the polymer is between approximately 10 and 80%; and the polymer is preferably adjusted to a predetermined pH value near the pI of the resulting conjugate with a buffer.

In a most preferred embodiment of the conjugate preparation method, the molecule containing a reactive aldehyde group is a polysaccharide, and the polysaccharide is first oxidized with an oxidizing agent or hydrolyzed. The oxidizing agent is preferably a glycol cleaving agent such as tetra-acetate, periodic acid, or sodium periodate. Unreacted oxidizing agent may be quenched with a quenching agent such as ethylene glycol. A protein is then added to the oxidized polysaccharide, and the polysaccharide and protein are coupled in the presence of a polymer or mixture of polymers to form polysaccharide-protein microparticles containing stable conjugates that form through a Schiff base intermediate. Additional reactive aldehyde groups on the conjugate may be quenched with a quenching agent such as hydroxamine.

Microparticles formed by the methods described herein may be administered to humans and other animals as microparticles by methods well known to those skilled in the art. Alternatively, the microparticles are substantially dissolved, yielding a composition containing soluble conjugate. The microparticles may be dissolved using chemical means,

such as with a solution adjusted to the appropriate pH, or by physical means, such as by sonication.

In accordance with the second preferred conjugate particle preparation method, which is useful for conjugate purification, conjugate particles are formed as described above and are separated from the unreacted components of the conjugation reaction mixture by physical means, yielding purified conjugates. In the third preferred embodiment, conjugates are formed by methods known to those skilled in the art, the conjugates are formed into particles by reacting the conjugates with a polymer or mixture of polymers as described above, and the particles are separated from the unreacted components of the conjugation reaction mixture by physical means, yielding purified conjugates. Suitable separation means include filtration, centrifugation, decantation, aspiration, and sedimentation. The particles may be washed for removal of additional undesired contaminants. The particles may then be solubilized, yielding soluble conjugates, prior to use.

The conjugates are prepared and purified in two or more separate steps or the conjugates are simultaneously prepared and purified.

Accordingly, it is an object of the present invention to provide a method for the preparation of macromolecular conjugates that is rapid, inexpensive, and efficient.

It is a further object of the present invention to provide a method for the preparation of stable, immunogenic conjugates.

It is a further object of the present invention to provide a rapid, inexpensive, and efficient method for the purification of conjugates.

It is a further object of the present invention to provide a rapid, inexpensive, and efficient method for the preparation and purification of carbohydrate-protein conjugates for use as conjugate vaccines.

It is a further object of the present invention to provide a method of separating conjugated polysaccharide from free polysaccharide having a very similar molecular weight.

It is a further object of the present invention to provide a method of separating conjugated protein from free protein having a very similar molecular weight.

These and other objects of the present invention will become apparent after reading the following detailed description of the disclosed embodiments and the appended claims.

Detailed Description of the Preferred Embodiments

Methods for preparing macromolecular conjugates and for purifying macromolecular conjugates from chemical reaction mixtures and the macromolecular conjugate produced by such methods are described herein. Conjugates are prepared by reacting the components to be conjugated or purified with a polymer or mixture of polymers to form conjugate particles. Conjugates form contemporaneously with the formation of the particles in the absence of reductive amination. Conjugates are purified by forming conjugate particles and separating the particles from non-reacted components. Conjugate particles are formed in the presence or absence of a conjugating reagent, depending on the nature of the components and the type of conjugation reaction employed. If desired, the conjugate particles may be dissolved chemically or physically to produce solubilized conjugates.

The term "macromolecular conjugate", as used herein, is defined as a biological or chemical molecule or macromolecule, which has been chemically conjugated to one or more biological or chemical molecules or macromolecules. The components of the conjugate may be molecules of the same type, such as protein-protein conjugates, or molecules of different types, such as carbohydrate-protein conjugates. The biological or chemical molecules or macromolecules used as components of the conjugates may be, but are not limited to, proteins, peptides, carbohydrates, glycoproteins, catecholamines, transmitters, hormones, receptors, labels, nucleic acids and chemicals.

The term "particles" as used herein is interchangeable with the terms "microparticles", "microspheres", and "microcapsules", and refers to solid or semi-solid particles having a diameter of less than one millimeter, more preferably less than 100 microns, and most preferably 10 microns or less.

The term "Schiff base" is defined herein as any of a class of derivatives of the condensation of aldehydes with primary amines. Schiff

bases are also referred to as imines. A Schiff base intermediate is a reversible condensation product of an aldehyde with an amine.

The term "polymer" as used herein includes a single polymer or a mixture of two or more polymers. It will be understood by those skilled in the art that a mixture of polymers may be premixed and added to the reaction mixture or the polymers may be added to the reaction mixture sequentially.

Conjugate Particle Formation

Conjugate particles are formed by reacting the components to be conjugated with a polymer. In a first preferred embodiment, a reaction mixture containing a molecule having a reactive amine group, a molecule having a reactive aldehyde group, and a polymer or mixture of polymers is reacted for a sufficient amount of time to form conjugate particles. In a second preferred embodiment, the components to be conjugated may be any molecules having the ability to be conjugated and are reacted with the polymer in the presence of a conjugating agent or agents to form conjugate particles. In a third preferred embodiment, pre-formed conjugates are reacted with the polymer to form conjugate particles. The latter two embodiments will be as described in more detail below with regard to conjugate purification.

Preferably, the reaction mixture is incubated at one or more temperatures between approximately 20° C and 65° C for a period of time between approximately 15 minutes and 5 hours at a pH near the pI of the resulting conjugate, preferably within one or two pH units of the pI of the conjugate. Most preferably, the reaction components are incubated at a temperature between room temperature and 58°C or at a series of different incubation temperatures within this range for a sufficient amount of time to allow formation of particles. The most preferred length of incubation time is between 30 minutes and 2 hours. The particles are then separated from the non-reacted components of the reaction mixture and may be solubilized using either chemical or physical means, yielding soluble conjugate as described in more detail below.

An organic solvent may be added to the reaction mixture to facilitate particle formation. The preferred organic solvent is an alcohol, such as ethanol, isopropanol, or methanol. Ethanol is the preferred alcohol.

A molecule having a reactive aldehyde group is defined herein as a molecule that either contains a reactive aldehyde group or can be modified to expose or otherwise include a reactive aldehyde group, such as by hydrolysis. A reactive aldehyde group is defined herein as an aldehyde group having the ability to react with and couple to an amine. A molecule having a reactive amine group is defined herein as a molecule that either contains a reactive amine group or can be modified to expose or otherwise include a reactive amine group. A reactive amine group is defined herein as an amine group having the ability to react with and couple to an aldehyde. The preferred molecule having a reactive aldehyde group is a polysaccharide that has been activated with a specified oxidizing agent or by hydrolysis, and the preferred molecule having a reactive amine group is a protein. When these preferred molecules are reacted with a polymer at room temperature or moderately elevated temperatures, polysaccharide-protein conjugate particles are formed. The preferred conjugates are both stable and immunogenic and are particularly useful as vaccines. Particles that are collected and washed prior to solubilization produce a purified conjugate vaccine preparation with excellent recovery of polysaccharide.

Each particle includes a non-resilient outer surface encapsulating an inner matrix. Both the outer surface and the inner matrix are composed of homogeneously distributed conjugated macromolecules and polymer molecules, which are intertwined. The inner matrix is water soluble, and the solubilized inner matrix diffuses through the outer surface. The particles have a generally uniform size and shape which, under most conditions, is between approximately 1 and 10 μm in diameter and is spherical. The outer surface of the particle is a hard, rigid, or non-resilient, solid material, most likely composed of denatured or semi-denatured macromolecules or conjugated macromolecules that resist solubilization, while the interior of the microparticle is more readily dissolved by aqueous solutions. The outer surface is penetrable and not only allows aqueous fluids to enter the interior of the particle, but also allows solubilized conjugate and polymer to exit the microparticle, resulting in a slow, sustained release of conjugate and polymer from the interior of the particle when placed in an appropriate aqueous medium.

The conjugates may be administered to a human or animal as particles, thereby providing a sustained or delayed release of the conjugate,

or the particles may be solubilized prior to administration, yielding soluble conjugates. Alternatively, a preparation containing a mixture of conjugate particles and solubilized conjugate may be formulated, resulting in both an immediate and a delayed or sustained release of conjugate. If the conjugate is a conjugate vaccine administered to a human or animal, the mixture of solubilized conjugate and conjugate particles is one that preferably contains a sufficient amount of soluble antigen to prime an immune response and contains a sufficient amount of slow-releasing antigenic particles to subsequently boost the immune response in a single administration.

In the preferred embodiment, the conjugate particles are substantially dissolved with an acid or a base or in a solubilizing solution, such as an acidic or alkaline solution, to produce a soluble conjugate. The conjugate may then be diluted with a physiologically acceptable buffer. If the pI of the conjugate is less than pH 7, then the conjugate particles are dissolved with a base or basic solution. The preferred base is sodium hydroxide. If the pI of the conjugate is greater than pH 7, then the conjugate particles are dissolved with an acid or acid solution. The preferred acid is hydrochloric acid. The pH of the dissolving solution for each conjugate particle preparation may be determined by titration, using techniques well known to those skilled in the art. The solubilizing solution is preferably dilute or at a minimum concentration, thereby reducing the amount of denaturation or destruction of the conjugate or the components of which the conjugate is made. In addition to the chemical dissolution methods described above, particles may be dissolved by physical methods such as sonication.

The conjugation method described herein is superior to methods currently employed, such as reductive amination, in that conjugation is achieved with a shorter production time, resulting in a higher yield of a substantially pure conjugate. Production is therefore significantly less expensive. In addition, this conjugate preparation method is particularly useful for the preparation of conjugates from macromolecules unable to withstand the harsh conditions of conventional conjugation procedures.

The conjugate purification method described herein is superior to known conjugate purification processes, such as gel filtration, ultrafiltration, ion exchange chromatography or mixtures thereof, in that the reagents are less expensive and the purification procedure is much more

rapid, taking only two to three hours rather than three to four days. The purification method described herein also permits purification of larger samples and rapid scale-up because it is not limited by column capacity. The purification method is particularly useful for the preparation, separation and isolation of carbohydrate-protein conjugates from free carbohydrates, especially when the carbohydrate-protein conjugate has a molecular weight very similar to the molecular weight of the carbohydrate prior to conjugation to the protein.

As discussed in more detail below, the conjugates are particularly useful as vaccines for administration to humans or animals, particularly infants, in which one or both of the unconjugated components have limited immunogenicity. The conjugates are additionally useful for *in vitro* and *in vivo* research and for diagnostic procedures wherein a macromolecule is labeled with a detectable label, such as fluorescein or a radiolabel, and the labeled conjugate is added to a biological sample or cell culture for the analysis of macromolecular structure and function.

Particle Formation Mechanism

Although not wishing to be bound by the following mechanism, it is believed that the conjugate particles are formed by a "macromolecular crowding" or "volume exclusion" effect in which the polymers strip water away from macromolecules in the solution and force these molecules together. This crowding lowers the diffusion coefficient and increases the rates and degree of association of these macromolecules. Covalent bonds are then formed between different reactive components, such as the formation of a stabilized Schiff base linkage between the activated aldehyde on the polysaccharide and amino lysine residues on the protein during the formation of a polysaccharide-protein conjugate. The polymer, or mixture of polymers, therefore act as a macromolecular crowding agent, which is defined herein as a compound that attracts water and allows molecules to aggregate. The polymer appears to induce formation of multiple Schiff bases, producing a stable conjugate that precipitates as microparticles during phase separation. Unexpectedly, even after the particles are washed and dissociated with a mild acid or base, the conjugates are stable and appear to remain covalently coupled. This stability is evidenced by the fact that solubilized conjugates induce a T-dependent immune response in an animal that could result only from a

polysaccharide-protein conjugate and not from the individual, unconjugated components.

Conjugate Purification

In accordance with the purification method, conjugate particles are formed, as described above and in more detail below, and the particles separated from the conjugation reaction mixture, which includes the unconjugated components, by conventional means well known to those skilled in the art yielding purified conjugates. The conjugate should have a pI that is different from the pI of the free components, particularly the protein component of a carbohydrate-protein conjugate, thereby facilitating separation. Suitable separation means include, but are not limited to, physical separation techniques such as filtration, centrifugation, decantation, aspiration, and sedimentation. The particles may be washed with a conventional solvent, such as water or a buffer, after physical separation from the majority of the unreacted components for removal of additional undesired contaminants.

In a first preferred embodiment of the purification method, a reaction mixture containing the components to be conjugated is reacted with a polymer or mixture of polymers for a sufficient amount of time to form conjugate particles. In other words, in the first preferred embodiment, the components are conjugated and formed into particles either simultaneously or immediately thereafter because the polymer is present as a component of the conjugation reaction mixture. The components may be a molecule containing a reactive aldehyde group and a molecule containing a reactive amine group as described above, or the components may be other molecules capable of being conjugated with a conjugating agent in accordance with methods known to those skilled in the art. Preferably, if a molecule containing the reactive aldehyde group, such as a carbohydrate, is one of the components to be conjugate, it is provided in excess so that all of the molecule containing the reactive amine group in the reaction mixture is conjugated.

In a second preferred embodiment of the purification method, pre-formed conjugates, which have been produced by the methods described herein or by methods known to those skilled in the art, are incubated with a polymer, or mixture of polymers, for a sufficient amount of time to form conjugate particles.

In both embodiments, the polymer and conjugates or conjugation reaction mixture is preferably incubated at one or more temperatures between approximately 20° C and 65° C for a period of time between approximately 15 minutes and 5 hours.

Conjugation Reactions

The conjugation reactions utilized in the preparation of conjugates that are then purified in accordance with the methods described herein are well known to those skilled in the art and include, but are not limited to, diazo-coupling; triazine bridge; thioether bond; amidation; isourea bond; aldimines; thiocarbamoyl formation; reductive amination reactions, such as described in U.S. Patent No. 4,356,170 to Jennings *et al.*, which is incorporated by reference herein; carbodiimide reactions; glutaraldehyde condensation reactions and other reactions including bisdiazotized benzidine; bisdiazotized phenylacetic acid; thioether reactions as described by Marburg *et al.*, "Bimolecular chemistry of macromolecules: Synthesis of bacterial polysaccharide conjugates with *Neisseria* meningitis membrane protein", *J. Am. Chem. Soc.* 108:5282-5287 (1986), which is incorporated by reference herein; and toluene diisocyanate reactions.

The conjugating agents useful for producing conjugates by the conjugation reactions described above utilize or modify naturally-occurring functional groups such as carboxyl, hemiacetal, amino/imino, mercapto/disulfide, hydroxyl, and phenoxyl moieties. Exemplary conjugating agents include, but are not limited to, bromide, thiol, hydrazide, cyanate, carboxyl, aldehydes, esters, boranes, and amines; more specifically cyanoborohydride, diazonium, triazine, isothiocyanate, aldonic acid, adipic acid dihydrazide, pyridyldisulfide, carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide hydrochloride (EDC), hydroxysuccinimide (NHS), sulfonated hydroxysuccinimide (sulfo-NHS), glutaraldehyde, pyridine borane, methyl borane, carbonyldiimidazoles, and derivatives thereof. Conjugating regents are commercially available from chemical companies such as the Sigma Chemical Company (St. Louis, MO). Examples of these reagents and typical coupling reactions are described by Cruse, J.M and Lewis, R.E., Jr. (eds.), Conjugate Vaccines. *Contrib. Microbiol. Immunol.* 10:48-114, Basel, Karger (1989), which is incorporated by reference herein. It is understood that other conjugating agents and methods not specifically mentioned herein, which are known to

one of ordinary skill in the art, may all be used in the practice of this method.

Conjugation reactions using linkers, such as carbonyl or imino groups, are also included within the scope of this method. Linkers may improve conjugation efficiencies, promote improved antigenicity for the conjugated components, physically separate the conjugated components, and serve as "spacers" to permit increases in translational and rotational characteristics of the components, thereby increasing access of binding sites to soluble ligands.

The purification method described herein will separate the conjugates from various coupling reagents including: chemical conjugating agents, chemical cross-linkers, such as heterobifunctional and homobifunctional cross-linkers including activated halogens, maleimides, pyridyl disulfides, and succinimidyls; and photoactivatable cross-linkers such as aryl azides. Reaction conditions, cross-linking compounds, and conjugation methods are described in chapter 38, pp. 605-618, and chapter 42, pp. 665-678 of the text NEUROENDOCRINE PEPTIDE METHODOLOGY, edited by P. Michael Conn, Academic Press, New York, (1989) which are incorporated by reference herein.

The conjugate particles are separated from the other reagents in the incubation mixture by conventional methods well known to those skilled in the art such as centrifugation, filtration or decantation in combination with established washing procedures. The resulting purified conjugate particles may then be resuspended in a physiologically-acceptable buffer such as a saline solution or phosphate buffered saline. The purified conjugates may be administered as microparticles. Alternatively, the purified particles may be dissolved with solubilizing solvent having a predetermined pH as described above.

Polymer Characteristics

Various polymers may be utilized to form the macromolecular conjugate particles. These polymers may be water soluble, semi-water soluble, or non-water soluble and can include the following categories of polymers: 1) carbohydrate-based polymers, such as methylcellulose, carboxymethyl cellulose-based polymers, dextran, polydextrose, chitins, chitosan, and starch, and derivatives thereof; 2) polyaliphatic alcohols such as polyethylene oxide and derivatives thereof including polyethylene glycol

(PEG), PEG-acrylates, polyethyleneimine, polyvinyl acetate, and derivatives thereof; 3) poly(vinyl) polymers such as poly(vinyl) alcohol, poly(vinyl)pyrrolidone, poly(vinyl)phosphate, poly(vinyl)phosphonic acid, and derivatives thereof; 4) polyacrylic acids and derivatives thereof; 5) polyorganic acids, such as polymaleic acid, and derivatives thereof; 6) polyamino acids, such as polylysine, and polyimino acids, such as polyimino tyrosine, and derivatives thereof; 7) co-polymers and block co-polymers, such as poloxamer or Pluronic L-101™ polymer, and derivatives thereof; 8) tert-polymers and derivatives thereof; 9) polyethers, such as poly(tetramethylene ether glycol), and derivatives thereof; 10) naturally occurring polymers, such as zein, chitosan and pullulan, and derivatives thereof; 11) polyimids, such as poly n-tris(hydroxymethyl)methylmethacrylate, and derivatives thereof; 12) surfactants, such as polyoxyethylene sorbitan, and derivatives thereof; 13) polyesters such as poly(ethylene glycol)(n)monomethyl ether mono(succinimidyl succinate)ester, and derivatives thereof; 14) branched and cyclo-polymers, such as branched PEG and cyclodextrins, and derivatives thereof; and 15) polyaldehydes, such as poly(perfluoropropylene oxide-b-perfluoroformaldehyde), and derivatives thereof.

The polymer utilized to form the conjugate particles is preferably polyvinylpyrrolidone, polyethylene glycol, dextran, polyoxyethylene-polyoxypropylene copolymer, polyvinyl alcohol, or mixtures thereof, the characteristics of which are described in more detail below. The polymer or polymer mixture may be prepared in accordance with the methods set forth in co-pending U.S. Patent Application Serial No. 07/817,610 filed January 7, 1992 by James E. Woiszwillo, or PCT Patent Application No. US93-00073, filed January 7, 1993 by James E. Woiszwillo, both of which are incorporated herein by reference, in which the polymer is dissolved in water or an aqueous solution, such as a buffer, in a concentration between approximately 1 and 50 g/100 ml depending on the molecular weight of the polymer. The preferred total polymer concentration in the polymer solution is between 10% and 80%, expressed as weight/weight %. The preferred concentration of each polymer in the polymer solution is between 20% and 30% The preferred pH of the polymer solution depends on the pI of the molecule being conjugated or the final conjugate, preferably within

one or two pH units of the pI. For most molecules, the pH of the polymer solution will be approximately between pH 4 and pH 7, most preferably pH 5. The pH may be adjusted during the preparation of the polymer solution by preparing the polymer in a buffer having a predetermined pH. Alternatively, the pH may be adjusted after preparation of the polymer solution with an acid or a base.

Polyoxyethylene-polyoxypropylene copolymer, also known as poloxamer, is sold by BASF (Parsippany, New Jersey) and is available in a variety of forms with different relative percentages of polyoxyethylene and polyoxypropylene within the copolymer.

PVP is a non-ionogenic, hydrophilic polymer having a mean molecular weight ranging from approximately 10,000 to 700,000 and the chemical formula $(C_6H_9NO)_n$. PVP is also known as poly[1-(2-oxo-1-pyrrolidinyl)ethylene], Povidone™, Polyvidone™, RP 143™, Kollidon™, Peregal ST™, Periston™, Plasdone™, Plasmosan™, Protagent™, Subtosan™, and Vinisil™. PVP is non-toxic, highly hygroscopic and readily dissolves in water or organic solvents.

Polyethylene glycol (PEG), also known as poly(oxyethylene) glycol, is a condensation polymer of ethylene oxide and water having the general chemical formula $HO(CH_2CH_2O)_nH$.

Dextran is a term applied to polysaccharides produced by bacteria growing on a sucrose substrate. Native dextrans produced by bacteria such as *Leuconostoc mesenteroides* and *Lactobacteria dextranicum* usually have a high molecular weight.

Polyvinyl alcohol (PVA) is a polymer prepared from polyvinyl acetates by replacement of the acetate groups with hydroxyl groups and has the formula $(CH_2CHOH)_n$. Most polyvinyl alcohols are soluble in water.

PEG, dextran, PVA and PVP are commercially available from chemical suppliers such as the Sigma Chemical Company (St. Louis, MO).

Most preferably, the polymer is a polymer mixture containing a solution of polyvinylpyrrolidone and polyethylene glycol. The polyvinylpyrrolidone has a molecular weight between approximately 10,000 and 360,000, most preferably 40,000. The polyethylene glycol has a molecular weight between approximately 2,000 and 8,000, most preferably 3,350. In the preferred embodiment, 25 g/100 ml of a 40,000 MW solution of polyvinylpyrrolidone (PVP) is mixed with a 25 g/100 ml

of a 3,350 MW solution of polyethylene glycol (PEG) to form a PVP/PEG polymer mixture.

Equal concentrations of PVP and PEG generally provide the most favorable polymer mixture for the formation of a polysaccharide-protein conjugate. The volume of polymer added to the polysaccharide varies depending on the sizes and quantities of the polysaccharide and protein. Preferably, approximately three volumes of the polymer mixture are added to one volume of a solution containing the polysaccharide and protein. The pH of the macromolecular crowding agent is preferably between 4 and 9, most preferably pH 5.

Particle Characteristics

As discussed above, the preferred molecule containing a reactive amine group is a peptide or protein having a tertiary structure or a molecule that can be coupled or coordinated with other molecules to create a molecule having a tertiary structure. The protein portion of the such a conjugate may be any type of protein that can be conjugated and specifically includes, but is not limited to, proteins derived from animals, plants, viruses, bacteria, fungi, and protozoa; including toxoids, toxins, exotoxins, antibodies, both monoclonal and polyclonal, receptor proteins, protein hormones, transporter proteins, such as hemoglobin, myoglobin and transferrin, enzymes, repressor proteins, stimulator proteins, growth factor proteins, structural proteins, such as muscle proteins, collagen, elastin, proteoglycans and fibronectin, recombinant proteins, and synthetic proteins.

As mentioned above, the molecule to which the protein is conjugated can be any molecule that will chemically conjugate to the protein in the presence of one or more conjugating reagents, including a second protein, a carbohydrate, glycoprotein, peptide, catecholamine, transmitter, hormone, receptor, label or chemical. Some specific examples are set forth below.

Protein-Protein, Peptide-Peptide and Peptide-Protein Conjugates

Various methods of conjugating small peptides, for example luteinizing hormone-releasing hormone, and thyrotropin releasing hormone, to larger proteins such as keyhole limpet hemocyanin, serum albumin, and thyroglobulin, are well known to those of average skill in the art and are especially useful to increase immunogenicity. Such conjugation

methods utilize certain functional groups on specific amino acids of both components with coupling reagents that specifically favor reaction at these groups. Many of these coupling reagents are divalent and thereby facilitate conjugation of the peptide or protein of interest to another peptide or protein containing similar functional groups. Functional groups at which a peptide or protein may be conjugated include, but are not limited to, amino, imino, amide, phenolic, carboxyl, imidazo, phenyl, guanidino, indolyl, sulfhydral, thiol, alcohols, and hydroxyl. In addition to the coupling reagents specified above, coupling reagents for protein-protein and peptide-protein conjugates may be selected from the following list, which is not limiting: dialdehydes such as glutaraldehyde and formaldehyde, carbodiimide, bisdiazotized benzidine, bisdiazotized phenylacetic acid, toluene and diisocyanate. In addition to direct methods of conjugation, chemical and photoactivatable cross linking methods are commonly used. These methods are described in greater detail in Chapter 38, pages 605 - 617, "Strategies for the preparation of haptens for conjugation and substrates for iodination for use in radioimmunoassay of small oligopeptides" by W.W. Youngblood and J.S. Kizer, and in Chapter 42, pages 665 - 678, "Preparation and use of specific polyclonal and monoclonal antibodies for immunohistochemistry of neuropeptides" by L. Jennes and W.E. Stumpf, both published in NEUROENDOCRINE PEPTIDE METHODOLOGY, P. M. Conn ed. Academic Press, New York, 1989, which are incorporated by reference herein.

<u>Carbohydrate-Protein Conjugates</u>

As discussed above, the preferred molecule containing a reactive aldehyde group is a carbohydrate. The carbohydrate portion of the carbohydrate-protein conjugate includes lipopolysaccharides, polysaccharides and oligosaccharides derived from organisms, such as, but not limited to, bacterial groups including Micrococcaceae, particularly the genera *Sarcina, Micrococcus* and *Staphylococcus*; Streptococcaceae, particularly the genera *Streptococcaceae* more particularly *S. pneumoniae* and other pneumococci *and Leuconostoc*, and more particularly the organisms classified as beta-hemolytic streptococci and Group B streptococci; Lactobacillaceae, particularly the genera *Lactobacillus*; Propionibacteriaceae, particularly the genera *Lactobacillus*,

Propionibacterium, Corynebacterium, Listeria, and *Erysipelothrix*; Bacillaceae, particularly the genera *Bacillus* and *Clostridium*; Neisseriaceae, particularly the genera *Neisseria*, more particularly the organisms *N. meningitidis* and *N. gonorrhoeae* and other meningococci; Brucellaceae, particularly the genera *Brucella, Bordetella, Pasteurella, Hemophilus*, more particularly the organisms *H. influenzae* and *H. influenzae* type b; *Escherichia*, more particularly the organism *E. coli*; *Erwinia, Shigella, Salmonella, Proteus, Yersinia, Enterobacter, Klebsiellae*, more particularly the organism *K. pneumoniae*, and *Serratia*; Pseudomonadaceae, particularly the genera *Pseudomonas*, more particularly *P. aeruginosa*, and *Acetobacter*; Spirillaceae, particularly the genera *Photobacterium, Zymomonas, Aeromonas, Vibrio*, more particularly the organism, *Vibrio cholerae*, *Desulfovibrio*, and *Spirillum*; the order Actinomycetales, particularly the genera, *Mycobacterium*, more particularly the organisms *M. tuberculosis, M. leprae, M. kansasii, M. avium*, and other mycobacteria isolates; *Actinomyces, Nocardia, Streptomyces*, the order Spirochetales, particularly the genera *Treponema, Borrelia, Leptospira, Spirocheta;* the order Mycoplasmataceae, particularly the genera *Mycoplasma*; Rickettsiaceae, particularly the genera *Rickettsia, Coxiella*; Chlamydiaceae, particularly the genera *Chlamydia*; and Bartonellaceae, particularly the genera *Bartonella*.

In addition, the carbohydrate portion of a carbohydrate-protein conjugate includes lipopolysaccharides, polysaccharides and oligosaccharides derived from organisms such as, but not limited to, fungal groups, represented by the following organisms, *Cryptococcus*, particularly *Crytococcus neoformans, Coccidiodes immitis, Histoplasma capsulatum, Blastomyces dermatitidis, Candida*, particularly *C. albicans, Aspergillus*, particularly *A. fumigatus*, Phycomycetes, particularly *Mucor* and *Rhizopus, Sporothrix schenckii, Microsporum* species, *Trichophyton* species, and *Epidermophyton floccosum*.

Furthermore, the carbohydrate portion of the carbohydrate-protein conjugate includes lipopolysaccharides, polysaccharides and oligosaccharides derived from organisms such as, but not limited to, protozal groups and virally associated lipopolysaccharides, polysaccharides and oligosaccharides.

Suitable polysaccharides include, but are not limited to, Pn23F, a pneumococcal type 23 F polysaccharide and Pn14, a pneumococcal type 14 polysaccharide, both available from the American Type Culture Collection (ATCC, Rockville, MD); and the polyribosylribitol phosphate (PRP) of *Haemophilus influenzae* type b (Hib). Additional polysaccharides include pneumococcal type 9V polysaccharide (Pn9V), pneumococcal type 4 polysaccharide (Pn4), pneumococcal type 18F polysaccharide (Pn18F) and pneumococcal type 18C polysaccharide (Pn18C).

More particularly with regard to the carbohydrate-protein conjugates, the protein portion includes, but is not limited to, tetanus toxoid, diphtheria toxoid, *Neisseria meningitidis* outer membrane protein, nontoxic cross-reacting mutant of diphtheria toxin, proteins derived from bacteria, exotoxins, and recombinant derivatives thereof, and synthetic proteins, preferably those containing lysine residues. For example, the cross-reactive material or "CRM" protein of diphtheria toxin, specifically the CRM_{197} protein, is suitable. CRM_{197} is a native mutant form of the diphtheria toxin. CRM_{197} is non-toxic due to a single amino acid change in the fragment A of the molecule. CRM_{197} is commercially available from commercial sources such as Sigma Chemical Co. (St. Louis, MO).

Preparation of Polysaccharide-Protein Conjugates

In a preferred embodiment of the conjugate preparation method, the conjugate formed by the method is a polysaccharide-protein conjugate. The polysaccharide of the polysaccharide-protein conjugate is one that includes an oxidizable terminal aldehyde group capable of reacting with an amino group of a protein to form a Schiff base intermediate. Alternatively, the polysaccharide is one onto which an aldehyde group can be introduced, preferably by hydrolysis. The polysaccharide is preferably a bacterial antigen capable of inducing an immune response when coupled to a protein carrier. Useful polysaccharides include, but are not limited to, those derived from *Haemophilus influenza*, pneumococci, meningococci, ß-hemolytic streptococci, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella*, and *Vibrio cholerae*. Most preferably, the polysaccharide is one that is incapable of inducing an effective immune response in infants when administered alone, but produces a T lymphocyte-dependent immune response when coupled to a protein carrier and administered to infants.

The protein may be any physiologically tolerated protein having a free amino group. Preferred proteins include, but are not limited to, tetanus toxoid, diphtheria toxoid, *Neisseria meningitidis* outer membrane protein, nontoxic cross-reacting mutant of diphtheria toxin, a protein derived from bacteria, or a synthetic protein containing lysine residues. The protein may be derived from the same source as the polysaccharide.

In accordance with the method of the preferred embodiment, the polysaccharide is oxidized by incubating the polysaccharide with an oxidizing agent. Preferably, the incubation is performed at room temperature for a sufficient amount of time to cause oxidation, most preferably between 15 and 45 minutes. The oxidizing agent may be any glycol cleaving agent capable of introducing an aldehyde. Preferably, the glycol cleaving agent is an oxidizing agent such as lead tetra-acetate, periodic acid, or sodium periodate. Most preferably, the glycol cleaving agent is sodium periodate. The glycol cleaving agent is then quenched by the addition of a quenching reagent. Preferably, the quenching reagent is an alcohol containing a vicinal-hydroxyl group such as ethylene glycol.

The protein is added to the oxidized polysaccharide reaction mixture and incubated in the presence of a polymer or mixture of polymers. The polymer may be added to the polysaccharide either before or after the addition of the protein.

Protein-Label Conjugates

A frequently utilized conjugation procedure involves the labeling of a protein with a detectable biomolecule or chemical for purposes such as *in vivo* and *in vitro* diagnostics and laboratory research. Various types of labels and methods of conjugating the labels to the proteins are well known to those skilled in the art. Several specific labels are set forth below. The labels are particularly useful when conjugated to a protein such as an antibody or receptor.

For example, the protein can be conjugated to a radiolabel such as, but not restricted to, ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or ^{131}I. Detection of a label can be by methods such as scintillation counting, gamma ray spectrometry or autoradiography.

Bioluminescent labels, such as derivatives of firefly luciferin, are also useful. The bioluminescent substance is covalently bound to the protein by conventional methods, and the labeled protein is detected when

an enzyme, such as luciferase, catalyzes a reaction with ATP causing the bioluminescent molecule to emit photons of light.

Fluorogens may also be used to label proteins. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycocyanin, phycocyanin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector.

The protein can alternatively be labeled with a chromogen to provide an enzyme or affinity label. For example, the protein can be biotinylated so that it can be utilized in a biotin-avidin reaction, which may also be coupled to a label such as an enzyme or fluorogen. For example, the protein can be labeled with peroxidase, alkaline phosphatase or other enzymes giving a chromogenic or fluorogenic reaction upon addition of substrate. Additives such as 5-amino-2,3-dihydro-1,4-phthalazinedione (also known as Luminol™) (Sigma Chemical Company, St. Louis, MO) and rate enhancers such as p-hydroxybiphenyl (also known as p-phenylphenol) (Sigma Chemical Company, St. Louis, MO) can be used to amplify enzymes such as horseradish peroxidase through a luminescent reaction; and luminogeneic or fluorogenic dioxetane derivatives of enzyme substrates can also be used. Such labels can be detected using enzyme-linked immunoassays (ELISA) or by detecting a color change with the aid of a spectrophotometer. In addition, proteins may be labeled with colloidal gold for use in immunoelectron microscopy in accordance with methods well known to those skilled in the art.

The location of a ligand in cells can be determined by labeling an antibody as described above and detecting the label in accordance with methods well known to those skilled in the art, such as immunofluorescence microscopy using procedures such as those described by Warren and Nelson, *Mol. Cell. Biol.* 7: 1326-1337 (1987).

<u>Formulations</u>

Macromolecular conjugates, prepared or purified by the methods described herein, which are useful for administration as vaccines, can be formulated and packaged, alone or in combination with other antigens, using methods and materials known to those skilled in the art for vaccines. A carbohydrate-protein conjugate prepared or purified as described herein is preferably added to the composite vaccine normally administered to infants.

The carbohydrate-protein conjugate may be administered with an adjuvant in an amount effective to enhance the immunogenic response against the conjugate. At this time, the only adjuvant approved for use in humans has been alum (aluminum phosphate or aluminum hydroxide). Saponin and its purified component Quil A, Freund's complete adjuvant and other adjuvants used in research and veterinary applications have toxicities which limit their potential use in human vaccines. However, chemically defined preparations such as muramyl dipeptide, monophosphoryl lipid A, phospholipid conjugates, such as those described by Goodman-Snitkoff et al. *J. Immunol.* 147:410-415 (1991) and incorporated by reference herein, encapsulation of the conjugate within a proteoliposome as described by Miller et al., *J. Exp. Med.* 176:1739-1744 (1992) and incorporated by reference herein, and encapsulation of the protein in lipid vesicles, such as Novasome™ lipid vesicles (Micro Vescular Systems, Inc., Nashua, NH) may also be useful.

<u>Methods of Administration and Dose</u>

The conjugate may be packaged as a vaccine in a single dosage for immunization by parenteral (*i.e.*, intramuscular, intradermal or subcutaneous) administration or nasopharyngeal (*i.e.*, intranasal) administration. It will be understood by those skilled in the art that the vaccine may confer immunity upon a single administration or upon multiple administrations including a primary administration and one or more subsequent administrations or boosts.

The conjugate is most preferably injected intramuscularly into the deltoid muscle. The conjugate is preferably combined with a pharmaceutically acceptable carrier to facilitate administration. The preferred carrier is water or a buffered saline, with or without a preservative. The antigen may be lyophilized for resuspension at the time of administration or in solution.

The carrier may also be a polymeric delayed release system. Synthetic polymers are particularly useful in the formulation of a vaccine to effect the controlled release of antigens. For example, the polymerization of methyl methacrylate into spheres having diameters less than one micron has been reported by Kreuter, J., MICROCAPSULES AND NANOPARTICLES IN MEDICINE AND PHARMACOLOGY, M. Donbrow (Ed). CRC Press, p. 125-148.

Microencapsulation of the conjugate or conjugate particle will provide a controlled release. A number of factors contribute to the selection of a particular polymer for microencapsulation. The reproducibility of polymer synthesis and the microencapsulation process, the cost of the microencapsulation materials and process, the toxicological profile, the requirements for variable release kinetics and the physicochemical compatibility of the polymer and the antigens are all factors that must be considered. Examples of useful polymers are polycarbonates, polyesters, polyurethanes, polyorthoesters polyamides, poly (d,l-lactide-co-glycolide) (PLGA) and other biodegradable polymers. The use of PLGA for the controlled release of antigen is reviewed by Eldridge, J.H., et al., *Current Topics in Microbiology and Immunology*, 146:59-66 (1989).

The preferred dose for a human infant of average weight is a 0.5 ml injection containing a carbohydrate concentration of between 5 and 25 μg of the carbohydrate-protein conjugate. Based on this range, equivalent dosages for heavier body weights can be determined. It is possible that, due to the increased purity of the conjugate contained in the vaccine, a smaller dose may be administered without sacrificing immunogenicity.

It will be understood by those skilled in the art that a conjugate vaccine may additionally contain stabilizers such as thimerosal (ethyl(2-mercaptobenzoato-S)mercury sodium salt) (Sigma Chemical Company, St. Louis, MO) or physiologically acceptable preservatives.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Preparation of *Haemophilus Influenza* Type b Polysaccharide-Tetanus Conjugate

This experiment describes the preparation of *Haemophilus influenzae* type b-tetanus toxoid conjugates in the absence of reductive amination by forming Schiff base intermediate conjugate particles using a mixture of polymers. The effects of the pH of the polymer solution on conjugate recovery and ratio of the conjugated components, immunogenicity of both the conjugate particles and solubilized conjugate,

and the effects of solubilizing base concentration on immunogenicity were analyzed.

General Conjugate Preparation Protocol

An aliquot containing 0.204 ml of purified polyribosylribitol phosphate (PRP) polysaccharide of *Haemophilus influenzae* type b (Hib) (24.5 mg/ml) (Massachusetts Public Health Biologic Laboratory (Jamaica Plain, MA)) was mixed with 0.125 ml of sodium periodate (8 mM in deionized water) (Sigma Chemical Company, St. Louis, MO) and incubated at room temperature for 30 minutes. 10 μl of ethylene glycol (Sigma Chemical Company, St. Louis, MO) was added and incubated at room temperature for 30 minutes. To the mixture was added 5 mg of purified tetanus toxoid (6.9 mg/ml) and three volumes of a polymer mixture containing 25% polyvinylpyrrolidone (PVP, molecular weight 40,000), 25% polyethylene glycol (PEG, molecular weight 3,350), and sodium acetate buffer, pH 5.0, while vortexing. The starting ratio of polysaccharide to protein was approximately 1:1 by weight.

The reaction mixture was incubated either a) at room temperature for 30 minutes; b) at room temperature for 30 minutes and at 37°C for 30 minutes; or c) at room temperature for 30 minutes, at 37°C for 30 minutes, and at 58°C for 30 minutes. Microparticles formed. The mixture was centrifuged at 13,200 rpm for 20 minutes to pellet the microparticles. The supernatant was decanted and the microparticles washed twice with 1.0 ml of 25% ethanol and 1.0 ml of deionized water. The resulting *Haemophilus influenzae* type b-tetanus toxoid (Hib-TT or PRP-TT) conjugate microparticles were injected into mice as described below. Alternatively, microparticles were suspended in 1.0 ml of deionized water, substantially dissolved in 0.1 N NaOH, and diluted in phosphate buffer to produce a soluble Schiff base intermediate *Haemophilus influenzae* type b-tetanus toxoid (Hib-TT) conjugate and injected as follows. The conjugates were analyzed for Hib polysaccharide content using the Orcinol assay and for protein content using the BCA™ protein assay (Pierce, Rockford, IL). The Orcinol assay is specific for pentose type carbohydrates.

A 0.2 ml aliquot of a 12.5 μg/ml saline solution of the Hib-TT conjugate (based on polysaccharide content) microparticles or solubilized Hib-TT conjugate microparticles was injected subcutaneously into CD-1 female mice (13-15 g) (groups of 10 mice). A boost was injected on day

28. Blood samples were taken for immunogenicity analysis on days 28 and 42.

Effect of Polymer Solution pH

Conjugate particles were prepared according to the protocol set forth above with the polymer solution containing sodium acetate buffer, pH 5.0, and the reactants incubated at room temperature for 30 minutes, at 37°C for 30 minutes, and at 58°C for 30 minutes. Conjugates were also prepared with a polymer solution containing sodium acetate buffer, pH 4.0. The percent recovery and ratio of polysaccharide to protein in the conjugate particles are set forth in Table 1 below.

Table 1: Effect of Polymer pH on Conjugate Recovery and PRP/TT Ratio

Polymer pH	Recovery (%)		PRP/TT Ratio in Conjugate (w/w)
	PRP	TT	
5.0	5.1	43	0.12
4.0	18.1	39	0.45

The results indicate that a higher polysaccharide recovery and higher ratio of polysaccharide to protein is achieved at pH 4.0.

Immunogenicity of Conjugate Particles

Conjugate particles were prepared as described in the general protocol set forth above. Sample 49-71 was incubated at room temperature for 30 minutes. Sample 49-72 was incubated at room temperature for 30 minutes and at 37°C for 30 minutes. Sample 49-73 was incubated at room temperature for 30 minutes, at 37°C for 30 minutes, and at 58°C for 30 minutes. Samples C29 and C30 were prepared in the absence of sodium periodate. Sample C29 was prepared in the presence of glutaraldehyde. The controls contained two different doses of a Hib-TT conjugate prepared using the conjugating reagent 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide hydrochloride (a conjugation method known to those skilled in the art as the "EDC" or "EDAC" reaction). The PRP+TT sample was prepared in the absence of polymer. The samples were injected into mice as described above.

The immunogenicity of the particles was determined by measuring the geometric mean concentration (GMC) of IgG antibodies to PRP and TT

in mouse blood samples taken two weeks after administration of the boost dose (secondary injection). The results are set forth in Table 2 below.

Table 2: Immunogenicity of PRP-TT Conjugate Particles in CD-1 Mice

Sample	Dose (μg)		GMC (μg/ml)	
	PRP	TT	Anti-PRP	Anti-TT
49-71	2.5	30	2.0	1407
49-72	2.5	30	3.0	1701
49-73	2.5	30	2.5	1966
Control	2.5	6.4	2.1	737
Control	12	30	2.2	225
C29	2.5	6.3	0.2	385
C30	2.5	11.3	<0.05	851
PRP+TT	2.5	30	0.2	841

The results indicate that the conjugate particles are immunogenic. However, in the absence of sodium periodate treatment (oxidation of aldehyde groups on polysaccharide prior to incubation with protein), the particles lack immunogenicity.

Immunogenicity of Solubilized PRP-TT Conjugate

The PRP-TT particles prepared above as Sample 49-72 were dissolved in 0.1 N NaOH and neutralized with phosphate buffer (15 mM) to produce solubilized PRP-TT conjugate. The solubilized conjugate was injected into groups of ten mice as described above in accordance with the general protocol. The immunogenicity of the solubilized conjugate is set forth in Table 3 below.

Table 3: Immunogenicity of Solubilized PRP-TT Conjugate Particles in CD-1 Mice

Sample	Dose (μg)		GMC at 2 Week after 2° (μg/ml)	
	PRP	TT	Anti-PRP IgG	Anti-TT IgG
Solubilized	2.5	30	117	9199
Particle	2.5	30	3.0	1701
Control PRP-T	2.5	6.4	2.1	737
Control PRP-T	12	30	2.2	225
PRP + TT	2.5	30	0.2	841

The results indicate that the solubilized conjugate is more immunogenic than the conjugate particles for both the polysaccharide and

the protein. In addition, enhanced anti-PRP and anti-TT antibody production was observed.

Effect of Base Concentration on Conjugate Immunogenicity

PRP-TT conjugate particles, prepared as described above, using incubations at room temperature for 30 minutes, at 37°C for 30 minutes, and at 58°C for 30 minutes, were dissolved with different concentrations of sodium hydroxide ranging from 0.2 N to 0.001 N and injected into groups of ten mice as described above. The immunogenicity results are shown below in Table 4.

Table 4: Effect of Base Concentration on Immunogenicity of PRP-TT Conjugate in CD-1 Mice

Sample	[NaOH]	Dose (μg)		GMC at 2 Week after 2°	(μg/ml)
		PRP	TT	Anti-PRP IgG	Anti-TT IgG
1	0.2N	2.0	25	2.1	887
2	0.1N	2.0	25	1.8	520
3	0.05N	2.0	25	2.3	394
4	0.01N	2.0	25	2.1	2050
5	0.005N	2.0	25	4.2	3932
6	0.001N	2.0	25	9.3	1550
Control PRP-T		2.0	5.1	1.3	15
Control PRP-T		9.8	25	2.7	110
PRP+TT		2.0	25	0.2	213

The results indicate that different concentrations of base produce varying antibody responses to both PRP and TT. As the concentration of sodium hydroxide was decreased, both anti-PRP and anti-TT antibody production was enhanced. The anti-PRP antibody titer was approximately four times higher that the commercial control, and the anti-TT antibody titer was approximately ten times higher than the commercial control.

Example 2
Preparation of Pneumococcal Polysaccharide Type 6B-Tetanus Toxoid Conjugate Vaccines

This experiment describes the preparation of pneumococcal polysaccharide type 6B-tetanus toxoid conjugates in the absence of reductive amination by forming Schiff base intermediate conjugate particles using a mixture of polymers. The effects of sodium periodate

concentration, pH of the polymer solution, and the starting ratio of the components on conjugate recovery and ratio of the conjugated components; the reproducibility of the conjugate preparation method; and the immunogenicity of conjugates prepared under slightly different conditions were analyzed.

The Pn6B polysaccharide is a pneumococcal type 6 B polysaccharide, available from the American Type Culture Collection (ATCC, Rockville, MD). Pn6B-TT conjugates were prepared in accordance with the general procedure set forth in Example 1 above for the preparation of PRP-TT conjugates. A 1:1 ratio of polysaccharide to protein was reacted by combining 5 mg of Pn6B (30 mg/ml) with 5 mg of TT (28 mg/ml).

Oxidation of Pn6B Polysaccharide

The effect of the sodium periodate concentration on the recovery of Pn6B and TT in the conjugate and the effect of the sodium periodate concentration the ratio of Pn6B to TT in the conjugate was studied. The results are shown below in Table 5.

Table 5: Effect of Concentration of $NaIO_4$ on Recovery and Ratio of Polysaccharide and Protein in the Conjugate

$[NaIO_4]$ mM	Recovery of Pn6B	Recovery of TT	Ratio of Pn6B/TT (w/w)
10	22%	57%	0.39
8	19%	47%	0.42
4	23%	53%	0.42

The results indicate that the lowest concentration of sodium periodate (4 mM) provides the highest recovery and highest ratio of Pn6B to TT in the conjugate.

Effect of Polymer Solution pH

Pn6B-TT conjugate particles were prepared according to the protocol set forth above with the polymer solution containing sodium acetate buffer, pH 5.0. Conjugates were also prepared with a polymer solution containing sodium acetate buffer, pH 4.0. The percent recovery and ratio of polysaccharide to protein in the conjugate particles are set forth in Table 6 below.

Table 6: Effect of pH Polymer Solution on Recovery and Ratio of Polysaccharide and Protein in the Conjugation

pH	Recovery of Pn6B	Recovery of TT	Ratio of Pn6B/TT (w/w)
5.0	23%	53%	0.42
4.0	53%	107%	0.50

The results indicate that a higher polysaccharide recovery and higher ratio of polysaccharide to protein is achieved at pH 4.0.

Starting Ratio of Pn6B to TT

Pn6B-TT conjugates were prepared according to the protocol set forth above with the polymer solution containing sodium acetate buffer, pH 5.0, using starting ratios of Pn6B to TT in the reaction mixture of 1:1 and 1:4. The Pn6B-TT conjugates were also prepared with the polymer solution adjusted to pH 4.0, using the following starting ratios of Pn6B to TT: 2:1, 1:1, 1:1.5 and 1:2. The percent recovery and ratio of polysaccharide to protein in the conjugate particles are set forth in Table 7 below.

Table 7: Effect of Starting Ratio of Pn6B to TT on Recovery and Ratio of Polysaccharide and Protein in the Conjugation

pH	Starting Ratio of Pn6B/TT (w/w)	Recovery of Pn6B	Recovery of TT	Ratio of Pn6B/TT (w/w)
5.0	1:1	23	53	0.42
5.0	1:4	69	58	0.33
4.0	2:1	49	75	1.21
4.0	1:1	53	107	0.50
4.0	1:1.5	72	84	0.53
4.0	1:2	54	111	0.24

The results indicate that the starting ratio has very little effect on conjugates prepared at pH 5.0. In contrast, the starting ratio of polysaccharide to protein has a significant, dose dependent effect on conjugates prepared at pH 4.0.

Reproducibility of Pn6B-TT Conjugate Preparation Procedure

Three different lots of Pn6B-TT conjugates were prepared as described above using 4 mM sodium periodate as the oxidizing agent, a polymer solution adjusted to a pH of 4.0, and a 2:1 starting ratio of Pn6B to TT. The different lots were then analyzed for percent recovery and ratio of polysaccharide to protein in the conjugate particles. The results are set forth in Table 8 below.

Table 8: Reproducibility of Pn6B-TT Conjugate

Lot	NaIO4 (mM)	pH	Starting Ratio of Pn6B/TT (w/w)	Recovery of Pn6B	Recovery of TT	Ratio of Pn6B/TT (w/w)
78-16	4.0	4.0	2:1	49.3	74.7	1.21
78-16C	4.0	4.0	2:1	53.2	83.5	1.17
78-37-1	4.0	4.0	2:1	50:8	112	1.04

The results show that the percent recovery and ratio of polysaccharide to protein in the conjugate particles are not significantly different from lot to lot, therefore the conjugate preparation system is reproducible.

Immunogenicity of Pn6B-TT Conjugates

Pn6B-TT conjugate particles were prepared as described above using 10 mM or 4 mM sodium periodate as the oxidizing agent and a polymer solution adjusted to a pH of 4.0 or a pH of 5.0 as specified below. The particles were dissolved in 0.1 N sodium hydroxide and neutralized in 0.02 M phosphate buffer to produce solubilized Pn6B-TT conjugate. The solubilized conjugate was injected subcutaneously into CD-1 female mice (13-15 g) (groups of 10 mice). A primary boost was injected on day 28. A secondary boost was injected on day 42. Blood samples were taken for immunogenicity analysis on day 0, day 28 (4w), day 42 (6w) and day 56 (8w).

Sample Pn6B+TT contained unconjugated polysaccharide and protein and was administered in a dose containing 2.0 μg Pn6B and 2.0 μg TT. Sample 6433-1 was prepared by oxidizing the polysaccharide with 10 mM sodium periodate and forming particles with a polymer solution at pH 5.0 and was administered in a dose containing 2.0 μg Pn6B and 5.1 μg TT. Sample 6433-2 was prepared by oxidizing the polysaccharide with 4 mM

sodium periodate and forming particles with a polymer solution at pH 5.0 and was administered in a dose containing 2.0 μg Pn6B and 5.1 μg TT. Sample 6433-3 was prepared by oxidizing the polysaccharide with 4 mM sodium periodate and forming particles with a polymer solution at pH 4.0 and was administered in a dose containing 2.0 μg Pn6B and 1.2 μg TT. Sample 6433-4 was prepared by oxidizing the polysaccharide with 4 mM sodium periodate and forming particles with a polymer solution at pH 4.0 and was administered in a dose containing 2.0 μg Pn6B and 2.7 μg TT. Sample 9411 contained Pn6B-TT conjugates prepared without polymers and without the formation of particles. These conjugates were made using cyanogen bromide and the conjugating reagent 1-ethyl-3-(3-dimethylaminopropyl-carbodiimide hydrochloride (a conjugation method known to those skilled in the art as the "EDC" or "EDAC" reaction). Sample 9411 was administered in a dose containing 2.0 μg Pn6B and 5.1 μg TT.

The immunogenicities of the solubilized conjugates are set forth below in Table 9. The abbreviation R/T in the table represents the number of "responder mice" over the number of total mice in the group.

Table 9: Geometric Mean Concentration of IgG Antibodies to Pn6B-TT Conjugates in CD-1 Mice

Sample	TT IgG (μg/ml) (R/T)			Pn6B IgG (μg/ml) (R/T)		
	4w	6w	8w	4w	6w	8w
Pn6B	4.615	20.91	180.75	0.024	0.023	0.028
+TT	(10/10)	(10/10)	(10/10)	(0/10)	(0/10)	(0/10)
6433-1	1.905	163.42	316.42	0.024	0.817	0.146
	(10/10)	(10/10)	(10/10)	(0/10)	(2/10)	(8/10)
6433-2	0.609	13.01	104.14	0.025	0.493	0.212
	(8/10)	(5/10)	(10/10)	(0/10)	(5/10)	(10/10)
6433-3	0.778	8.888	38.85	0.338	1.646	3.650
	(5/10)	(10/10)	(10/10)	(4/10)	(10/10)	(10/10)
6433-4	0.784	19.058	87.65	0.236	0.784	0.957
	(5/10)	(10/10)	(10/10)	(5/10)	(8/10)	(10/10)
9411	6.980	15.861	74.736	0.873	0.558	0.937
	(10/10)	(10/10)	(10/10)	(7/10)	(8/8)	(8/8)

The results indicate that the sample prepared using 4 mM sodium periodate and polymers at pH 4.0 provided an immune response to Pn6B at 6 weeks and 8 weeks that was far superior to all of the other samples.

Example 3
Preparation of Pneumococcal Polysaccharide Type 23F-CRM197 Conjugate Vaccines

This experiment describes the preparation of pneumococcal polysaccharide type 23F-CRM_{197} conjugates in the absence of reductive amination by forming Schiff base intermediate conjugate particles using a mixture of polymers. The immunogenicity of the conjugates were analyzed in mice.

Pn23F polysaccharide is a high molecular weight polysaccharide having an approximate molecular weight of from 1 to 3 million. The Pn23F polysaccharide is a pneumococcal type 23 F polysaccharide and is available from the American Type Culture Collection (ATCC, Rockville, MD). The CRM_{197} protein is a native, non-toxic, mutant form of the cross-reactive material of diphtheria toxin. CRM_{197} is commercially available from Sigma Chemical Co. (St. Louis, MO).

Pn23F-CRM_{197} conjugates were prepared by first combining. a Pn23F polysaccharide solution (11.3 mg/ml) with a sodium periodate solution (2.5 mM) and incubating the reaction at room temperature for one hour. Ethylene glycol was then added and the reactants incubated a further 30 minutes at room temperature to give a quenched, oxidized polysaccharide solution.

Sample 1

A 146 µl aliquot of the quenched, oxidized polysaccharide solution (1.5 mg polysaccharide) was combined with 56 µl of CRM_{197} (1.0 mg) and mixed well. The mixture was diluted with phosphate buffered saline to produce a polysaccharide concentration of 12.5 µg/ml and a protein concentration of 8.3 µg/ml. Polysaccharide concentration was determined by the Anthrone free polysaccharide assay described in METHODS IN IMMUNOLOGY AND IMMUNOCHEMISTRY, Vol. II, Williams, C.A. and Chase, M.W.(eds.), 1968, pp. 288 - 289, Academic Press, NY. This sample served as a control in that it lacked the addition of polymer and therefore no particles and no conjugates were formed.

Sample 2

A 146 µl aliquot of the quenched, oxidized polysaccharide solution (1.5 mg polysaccharide) was combined with 56 µl of CRM_{197} (1.0 mg) and mixed well. To this solution was added 0.6 ml of a polymer mixture containing 25% PVP, 25% PEG, and sodium acetate buffer, pH 5.0, and the entire mixture was mixed using a syringe. 200 µl of ethanol was added and the solution mixed well and incubated at room temperature for 30 minutes, at 37°C for 30 minutes, and at 50°C for 30 minutes. The mixture was centrifuged at 13,200 rpm for 10 minutes and the pellet was washed three times with 0.5 ml of 30% ethanol. The pellet was resuspended in phosphate buffered saline to provide a polysaccharide concentration of 12.5 µg/ml.

CD-1 mice (4-6 weeks of age) were injected with two doses of the samples (2.5 µg polysaccharide) with the booster dose being given on day 28. As additional controls, mice were also injected with the Pn23F polysaccharide alone and the oxidized Pn23F polysaccharide alone. Blood samples were taken on day 42. The sera was diluted 200 and 800 fold and analyzed by ELISA for Pn23F. The results are presented in Table 10 below.

Table 10: Immunogenicity of Pn23F-CRM197 Conjugates

Sample	ELISA Absorbances	
	200-fold dilution	800-fold dilution
1	0.092	0.009
2	0.593	0.136
Pn23F	0.267	0.042
Pn23F (oxidized)	0.112	0.029

The results indicate that Sample 1, which contained a solution of Pn23F and CRM_{197} that was not treated with polymers, was non-immunogenic. In contrast, Sample 2, which contained a solution of Pn23F and CRM_{197} that was treated with polymers to produce conjugate particles, was immunogenic.

Example 4
Preparation of Pneumococcal Polysaccharide Type 18C-Tetanus Toxoid Conjugate Vaccines

This experiment describes the preparation of pneumococcal polysaccharide type 18C-tetanus toxoid conjugates in the absence of reductive amination by forming Schiff base intermediate conjugate particles using a mixture of polymers. The effects of the starting ratio of the components, the sodium periodate concentration, and the incubation times and temperatures on conjugate recovery and ratio of the conjugated components were analyzed.

The Pn18C polysaccharide is a pneumococcal type 18 C polysaccharide, available from the American Type Culture Collection (ATCC, Rockville, MD). Pn6B-TT conjugates were prepared in accordance with the general procedure set forth in Example 1 above for the preparation of PRP-TT conjugates. Pn18C was mixed with sodium periodate for 30 minutes and quenched with ethylene glycol. TT was added to the quenched mixture along with a polymer mixture containing 20% PVP and 20% PEG, pH 4. The mixture was incubated at room temperature, 37°C, and 58°C as indicated below in Table 11. Particles formed and were centrifuged and washed with deionized water. The particles were dissolved with sodium hydroxide and neutralized with phosphate buffer (pH 7).

The resulting conjugates all exhibited similar HPLC profiles showing a major conjugate peak and a very minor peak having the retention time of TT, regardless of the preparation conditions used.

The polysaccharide content was determined using the Anthrone free polysaccharide assay described in METHODS IN IMMUNOLOGY AND IMMUNOCHEMISTRY, Vol. II, Williams, C.A. and Chase, M.W.(eds.), 1968, pp. 288 - 289, Academic Press, NY. The protein content was determined using the BCA™ protein assay (Pierce, Rockford, IL). The HPLC analysis was performed on a TSK-4000 column. The results are shown below in Table 11.

Table 11: Recovery Yield and Ratio of Polysaccharide to Protein in Pn18C-TT Conjugates Prepared Under Various Conditions

Start Pn18C/ TT	[NaIO4] in reaction (mM)	Incubation time (hr) r.t.	37°	58°	Pn18C recovery yield (%)	TT recovery yield (%)	Pn18C/ TT in con- jugate
1.37	16	.5	.5	.5	105	106	1.36
1.37	8	.5	.5	.5	99	111	1.22
2	16	.5	.5	0	66	122	1.11
2	8	.5	.5	0	60	101	1.23
2	16	1	2	0	72	114	1.31
2	16	1	4	0	66	107	1.28
4	16	1	2	0	64	149	1.77
4	16	1	4	0	63	133	1.96
2	8	.5	1	0	52	80	1.28
2	2	.5	1	0	27	52	0.95
1	4	.5	1	0	75	80	0.91

The results indicate that the starting ratio of Pn18C to TT had little effect on the recovery yields, Pn18C/TT ratios of conjugates, and HPLC profiles. Therefore, the starting ratio had little effect on conjugation. The concentration of sodium periodate also had little effect on conjugation provided that the concentration was equal to or greater than 4 mM. Although incubation at the highest temperature (58°C) produced particles having an enhanced recovery yield of Pn18C in the conjugates, conjugate particles formed using this temperature could not be dissolved completely with the sodium hydroxide. The length of time of incubation failed to affect on conjugation. A higher recovery yield of Pn18C was achieved with the lowest starting ratio of Pn18C to TT.

Example 5
Preparation of Group A Meningococcal Polysaccharide-Tetanus Toxoid Conjugates

This experiment describes the preparation of Group A meningococcal polysaccharide (GAMP)-tetanus toxoid conjugates in the absence of reductive amination by forming Schiff base intermediate conjugate particles using a mixture of polymers. Several physicochemical characteristics of two preparations of the GAMP-TT conjugates were determined.

Group A meningococcal polysaccharide having a size of 96,000 D (Shanghai Institute of Biological Production, P.R. China) was dissolved in

0.1 M MES (2-[N-morpholino]ethanesulfonic acid) buffer, pH 5.0, and incubated at 60°C for one hour to produce hydrolyzed GAMP having a size of 64,000 D. To this solution, a sufficient amount of borate salt powder was added to provide 0.1 M borate buffer, pH 8.5. Subsequently, a sufficient amount of $NaBH_4$ was added to provide a final concentration of 0.2 M $NaBH_4$. The mixture was incubated at room temperature for two hours and dialyzed against deionized water for three days. The resulting reduced GAMP had a size of 50,000 daltons and a concentration of 8.7 mg/ml.

Sodium periodate was added to provide a concentration of 8 mM. The mixture was incubated in the dark at room temperature for 30 minutes, then quenched by adding ethylene glycol (to provide a final concentration of 50 mM).

TT (10 mg/ml) was added, followed by the addition of three volumes of a polymer solution containing 25% PVP, 25% PEG, and sodium acetate buffer, pH 4.0. The reaction mixture was incubated at room temperature for thirty minutes and 37°C for thirty minutes. The pellet was collected by centrifugation and divided into two parts.

Pellet 1 was resuspended in deionized water, allowed to stand for one hour, washed with deionized water three times, dissolved in 0.1 N sodium hydroxide, and neutralized with 0.1 N HCl to provide GAMP-TT conjugate solution 1.

Pellet 2 was suspended in 0.1 M hydroxamine (NH_2OH) at room temperature for one hour to quench unreacted aldehyde groups and washed with saturated ammonia sulfate three times. The pellet was dissolved in 9.2 M urea and dialyzed against 0.9% saline to provide GAMP-TT conjugate solution 2.

The polysaccharide content of the conjugates was determined using the phosphorus assay (*Anal. Chem.* 28:1756-1759 (1956)). The protein content of the conjugates was determined using the well known Bradford protein assay. The free polysaccharide content was determined using an acid-detergent precipitation method.

The results are set forth in Table 12 below.

Table 12: Physico-chemical Characteristics of GAMP-TT Conjugates

	GAMP-TT 1	GAMP-TT 2
Polysaccharide recovery	22.65%	18.5%
Protein recovery	56.4%	52.5%
Free polysaccharide	4.26%	0
Polysaccharide/ protein ratio	0.321	0.282

The results indicate that less free polysaccharide is produced using the method used to prepare GAMP-TT 2.

Example 6
Preparation of Group C Meningococcal Polysaccharide-Diphtheria Toxoid Conjugate Vaccines

This experiment describes the preparation of Group C meningococcal polysaccharide-tetanus toxoid conjugates in the absence of reductive amination by forming Schiff base intermediate conjugate particles using a mixture of polymers. The immunogenicity of the conjugate in rabbits was determined.

Conjugate Preparation

Meningococcal C polysaccharide (Connaught Laboratories, Swiftwater, PA) was dissolved in deionized water (20 mg/ml). To this solution (0.3 ml) was added sodium periodate (8 mM, 0.3 ml) in deionized water. The mixture was incubated at room temperature for 30 minutes. 12 µl of ethylene glycol was added and mixed well. The mixture was incubated at room temperature for 30 minutes. Diphtheria toxoid (3.9 mg/ml, 0.6 ml, Connaught Laboratories) was added while vortexing. To the oxidized polysaccharide and protein mixture was added a 50% polymer solution containing 25% PVP (40,000 MW), 25% PEG (3,360 MW), pH 5.0 in sodium acetate buffer (2.4 ml). This reaction mixture was incubated at room temperature for 30 minutes and at 37°C for 30 minutes. Particle formed and were centrifuged and washed with 25% ethanol (0.8 ml) and deionized water (twice using 0.8 ml). The particles were resuspended in deionized water (0.8 ml) and analyzed for protein content using the BCA™ protein assay (Pierce, Rockford, IL) and polysaccharide content using the sialic acid assay (*Biochimica Biophysica Acta* 24:604-611 (1957). The

ratio of polysaccharide to protein was 0.14. Alternatively, the particles were redissolved in 0.1 N NaOH and neutralized with phosphate buffer.

Immunogenicity of the Conjugate in Rabbits

A 0.5 ml aliquot of a 20 μg/ml (polysaccharide content) phosphate buffered saline solution of the conjugate microparticles or the solubilized conjugated microparticles was injected subcutaneously into New Zealand rabbits (two rabbits per group). A boost was injected on day 21 and 42. Blood samples were taken for immunogenicity analysis on days 0, 7, 21, 27, 35, 42, and 49. Group I was injected with the conjugate microparticles, whereas Group II was injected with the solubilized conjugate. The control was commercially available meningococcal C polysaccharide vaccine at the same dose as the conjugate. The results, shown in Table 13 below, demonstrate that the conjugates are highly immunogenic.

Table 13: Immunogenicity of Meningococcal C Polysaccharide-Diphtheria Toxoid Conjugates in Rabbits

Group	Animal No.	IgG ELISA Titer (1:100 D) OD 450 nm					
		Day 7	Day 21	Day 27	Day 35	Day 42	Day 49
I	1	1.35	1.67	2.44	1.53	1.69	1.80
	2	1.24	0.46	3.17	1.83	1.58	0.90
II	1	2.32	1.98	3.31	2.37	2.99	2.12
	2	0.51	0.77	3.11	2.22	2.80	1.62
Control	1	0.00	0.17	0.17	0.40	0.17	0.10
	2	0.00	0.11	0.00	0.03	0.09	0.02

Example 7
Use of Polymers to Purify Conjugates Prepared by Reductive Amination

The polysaccharide-protein conjugates Pn23F-CRM197 and Pnl4-CRM197 were purified as described below by forming conjugate particles and separating the particles from unreacted components and reagents in the conjugate reaction mixture.

Pn23F polysaccharide and Pnl4 polysaccharide are high molecular weight polysaccharides, each having an approximate molecular weight of from 1 to 3 million. The Pn23F polysaccharide is a pneumococcal type 23 F polysaccharide. The Pnl4 polysaccharide is a pneumococcal type 14 polysaccharide. Both polysaccharides are available from the American

Type Culture Collection (ATCC, Rockville, MD). The CRM_{197} protein is a native, non-toxic, mutant form of the cross-reactive material of diphtheria toxin. CRM_{197} is commercially available from Sigma Chemical Co. (St. Louis, MO).

Each polysaccharide was independently conjugated to the CRM_{197} protein using a reductive amination conjugation reaction. The Pn23F-CRM_{197} conjugate reaction mixture was carried out using dimethylsulfoxide (DMSO). The Pnl4-CRM_{197} conjugate reaction mixture was carried out using phosphate buffer.

Purification of the Pn23F-CRM_{197} and Pnl4-CRM_{197} conjugates from free polysaccharide was achieved in two to three hours by incubating the conjugates with a polymer mixture of polyvinylpyrrolidone and polyethylene glycol to form conjugate particles and the particles separated from the reaction mixture by centrifugation and decantation.

Purification of Pn23F-CRM 197 Conjugate

The conjugate reaction mixture containing the Pn23F-CRM_{197} conjugate in dimethylsulfoxide (DMSO) was diluted with deionized water to give a 50% DMSO solution. The diluted solution was then concentrated with a Centricon 30 filter (Amicon, Beverly, MA) to yield a solution containing 2.84 mg/ml CRM_{197}, determined by the BCA™ protein assay (Pierce, Rockford, IL). To the mixture (1.8 ml) was added a polymer solution containing 25% PVP (40 kD), 25% PEG (3.35 kD) in 0.1M sodium acetate, pH 5.0 (5.4 ml), while vortexing. To the polymer/conjugate mixture was added 2.7 ml of a 95% ethanol solution while vortexing. The mixture was incubated for 30 minutes each at 20°C, 37°C and 60°C. Conjugate particles were formed. The mixture was centrifuged at approximately 14,000 x *g* in a microfuge at room temperature to precipitate the particles, and the supernatant was removed.

The pellet, containing the conjugate particles, was washed three times at room temperature with 2.4 ml of 50% ethanol to remove free polysaccharide and residual polymers. The pellet was then resuspended in 3.5 ml of phosphate buffered saline (PBS) to dissolve the particles, thereby resulting in a solubilized conjugate. Polysaccharide content was determined by the Anthrone free polysaccharide assay described in METHODS IN IMMUNOLOGY AND IMMUNOCHEMISTRY, Vol. II, Williams, C.A. and Chase, M.W.(eds.), 1968, pp. 288 - 289, Academic Press, NY,

which is incorporated herein by reference. Protein content was determined by the BCA™ protein assay (Pierce, Rockford, IL). The polysaccharide content was calculated to be 1.47 mg/ml. The protein content was calculated to be 1.26 mg/ml. Recovery of the conjugate was calculated to be 86%, as determined by comparing the protein content of the conjugate reaction mixture after purification to the protein levels in the conjugate reaction mixture before purification. Free polysaccharide content in the purified conjugate mixture was determined by first passing the mixture through a phenyl-Sepharose™ column (Pharmacia Biotech, Piscataway, NY), which binds the conjugate. The flow-through of this column was tested for free polysaccharide content using the Anthrone free polysaccharide assay described above and was found to average approximately 17%.

Purification of Pnl4-CRM197 Conjugate

The conjugate reaction mixture containing the Pn14F-CRM_{197} conjugate in phosphate buffer (1.8 ml) was mixed with a polymer solution containing 25% PVP (40 kD) and 25% PEG (3.35 kD) in 0.1 M sodium acetate (1.8 ml), pH 5. The mixture was incubated at room temperature for one hour. Conjugate particles were formed. The mixture was centrifuged at approximately 14,000 x *g* at room temperature to collect the particles, and the supernatant was removed. The residue was washed with 25% ethanol (2.4 ml) and then washed twice with deionized water (2.4 ml). The pellet was then resuspended in 5.9 ml of phosphate buffered saline with sonication to yield the conjugate in solubilized form. The recovery of the conjugate was 90% as determined by comparing the protein content of the conjugate reaction mixture before and after purification. The free polysaccharide content was approximately 14% as determined by the Anthrone free polysaccharide assay described above.

Example 8
Immunogenicity Study of Purified Pneumococcal Conjugates

An immunogenicity study was performed in rabbits to determine whether the Pn23F-CRM_{197} and Pnl4-CRM_{197} conjugates, purified as described in Example 1 above, were capable of generating an appropriate immune response.

New Zealand rabbits, five per group, were vaccinated with a preparation of each of the purified conjugates (containing 25 μg of carbohydrate) at 0 and 4 weeks. Antibody (Ab) titer values were determined by ELISA and expressed as a geometric mean of dilution showing 2X background optical density (O.D.). Alum (aluminum phosphate) was used as an adjuvant.

The immunogenicity data is summarized in Table 14, below.

Table 14: Anti-Pneumococcal Antibody Responses in Rabbits Determined By ELISA

Sample	Alum	Ab Titer week 0	Ab Titer week 2	Ab Titer week 4
Pn14-CRM_{197}	+	<100	12,054	69,923
Pn23F-CRM_{197}	+	<100	<100	3,446

Example 9
Purification of PRP-AH-TT from the Carbodiimide Reaction

A PRP-AH-TT conjugate was prepared and purified using the following procedures.

Preparation of PRP-AH-TT Conjugate

An adipic acid dihydrazide derivative (AH) of the polyribosylribitol phosphate (PRP) of *Haemophilus influenzae* type b (Hib), one of the major causative organisms of bacterial meningitis, referred to as PRP-AH, was prepared by coupling PRP to adipic acid (Sigma Chemical Co., St. Louis, MO) in the presence of cyanogen bromide (Sigma Chemical Co., St. Louis, MO) (The PRP was obtained from the Massachusetts Public Health Biologic Laboratory (Jamaica Plain, MA)). PRP-AH was coupled to tetanus toxoid (TT) in the presence of 1-3-ethyl-3-(3 dimethyl aminopropyl) carbodiimide (EDC, Sigma Chemical Co., St. Louis, MO) to generate a PRP-AH-TT conjugate. In this reaction, a wide range of molecular sizes of the conjugate were formed.

When purified by the conventional method, *e.g.*, size exclusion column chromatography, only high molecular weights of the conjugate can be collected without contamination by the free polysaccharide, which has a molecular weight of approximately 200,000 daltons. Thus, significant amounts of low molecular weight species of the conjugate are wasted

during chromatographic purification. Furthermore, the conventional purification process requires several days to complete.

Purification of PRP-AH-TT conjugate

A reaction mixture (0.2 ml) containing PRP-AH (200,000 D) (10 mg/ml) and TT (10 mg/ml) conjugates was combined with a polymer solution (0.6 ml) containing 25% PVP and 25% PEG in 0.1M sodium acetate pH 5.0, while vortexing. The mixture was incubated sequentially at room temperature, 37°C, and 58°C for 30 minutes each. Conjugate particles were formed. The mixture was centrifuged in a microfuge at 14,000 x g and washed three times with deionized water (0.5 ml). The pellet was resuspended in 0.5 ml of deionized water. Next, 50 µl of 1N NaOH was added followed by 1.0 ml of 1M phosphate buffer, pH 7.0, and sonication for three seconds. Recovery of TT and PRP concentrations were 95% and 37%, respectively. As a control, the conjugate was purified using a Sepharose™ CL4B column (Pharmacia, Piscataway, NJ), with phosphate buffered saline, pH 7 as the eluant, 2 ml fractions were collected, and the first peak ($K_d<0.3$) was pooled. (The term K_d as used herein refers to a distribution coefficient, which is defined as the fraction of the stationary phase available for diffusion of a given solute species. K_d equals the elution volume (V_e) minus the void volume (V_o) divided by the total volume of the packed bed (V_t)). Recoveries of TT and PRP were 37% and 24%, respectively. In both cases HPLC analysis revealed no significant PRP peak.

Using the polymer purification method, all conjugate was precipitated and isolated from the reaction mixture in only two to three hours. The polymer purification process provides higher yield and lower cost than the conventional chromatography purification process described above.

Example 10
Simultaneous Carbodiimide Coupling and Conjugate Purification Using Polymers

PRP-AH-TT polysaccharide-protein conjugates, as defined in Example 9, were formed and purified in a single continuous process in the same reaction vessel using the following method. Various conjugation reaction conditions were studied in which the polymers were added either at the beginning of the conjugation reaction or at a particular time during the conjugation reaction.

In samples 1-3, a conjugation reaction mixture was prepared containing tetanus toxoid (TT, 10 mg/ml) in 0.2 M MES buffer (pH 5.0), an adipic acid dihydrazide derivative (AH) of the polyribosylribitol phosphate (PRP) of *Haemophilus influenzae* type b (Hib), (PRP-AH, 10 mg/ml, prepared as described in Example 3) in 0.2 M MES buffer (pH 5.0), and 0.1 M of the carbodiimide conjugating reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), Sigma Chemical Co., St. Louis, MO) in MES buffer (pH 5.0).

In the first sample (Sample 1), a polymer solution consisting of three volumes of conjugation mixture containing 25% PVP (40,000 D), 25% PEG (3,350 D) in 0.1 M MES buffer pH 5.0 was added at the beginning of the conjugation reaction. The mixture was incubated at room temperature for 30 minutes, at 37°C for 30 minutes, and at 58°C for 30 minutes. The particles were recovered and resuspended in 25% ethanol and centrifuged at 13,200 rpm for 5 minutes, and resuspended in dH_20 twice and centrifuged at 13,200 rpm for 5 minutes. The washed particles were then dissolved in 0.1 N NaOH and neutralized with phosphate buffer. The resulting conjugate was analyzed for PRP by Ribose assay, TT by BCA assay and unconjugated PRP and TT by HPLC using a TSK column, and the molecular distribution of the conjugate was analyzed by gel filtration chromatography (CL-4B).

In the second sample (Sample 2), a similar procedure was followed as for the first sample except the polymer solution was added after the conjugation mixture was incubated at room temperature for 1 hour.

In the third sample (Sample 3), the polymer solution was added after the conjugation mixture was incubated at room temperature for 3 hours.

The results of samples 1-3, as listed in Table 15, indicate that the polymer solution containing PVP and PEG can be used to separate PRP-AH-TT conjugates from the reaction mixture even though approximately 5% unconjugated PRP may exist in the conjugate preparation.

Table 15: PRP-AH-TT Conjugate Formation and Purification Using Carbodiimide Conjugation in the Presence of Polymers

	Sample 1	Sample 2	Sample 3
Time of Polymer Addition	0 hour	1 hour	3 hours
PRP/TT (w/w)	0.364	0.344	0.401
PRP Recovery (%)	22.7	29.3	33.2
TT Recovery (%)	62.5	85.2	82.8
Free TT	0	0	0
Free PRP	<5%	<5%	<5%

Example 11
Preparation of Purified FITC-labelled Albumin

Fluorescein isothiocyanate (FITC, Sigma Chemical Company, St. Louis, MO) was conjugated to human serum albumin (HSA) in the presence of polymers causing the formation of conjugate particles. Free FITC was washed away from the particles, and the particles redissolved in NaOH yielding FITC-labelled HSA in the absence of free FITC.

Procedure

FITC (6.2 μg) was dissolved in 2 ml of carbonate buffer (pH 10). The dissolved FITC was combined with 1 ml of HSA (25%) and 6 ml of a polymer solution containing 25% PVP and 25% PEG in 0.1M sodium acetate pH 5.0, while vortexing. The mixture was incubated sequentially at room temperature, 37°C, and 58°C for 30 minutes each. Conjugate particles were formed. The mixture was centrifuged in a microfuge at 14,000 x g, the supernatant removed, and the particles washed three times with deionized water (10 ml each). The particles were resuspended in 10 ml deionized water.

The resuspended particles were visualized in a fluorescence microscope. All fluorescence was associated with the particles. No free fluorescence was observed, indicating that all of the FITC was conjugated to albumin and was not free FITC.

Modifications and variations of the conjugate preparation and purification methods will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

Claims

We claim:

1. A method for preparing macromolecular conjugates comprising reacting a reaction mixture comprising a molecule having a reactive amine group, a molecule having a reactive aldehyde group, and a polymer for a sufficient amount of time to form conjugate particles, wherein the conjugate particles are comprised of the macromolecular conjugates.

2. The method of claim 1 further comprising separating the particles from the reaction mixture.

3. The method of claim 1 further comprising solubilizing the particles.

4. The method of claim 1 wherein the polymer is a compound selected from the group consisting of carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers and block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched and cyclo-polymers, and polyaldehydes, and mixtures thereof.

5. The method of claim 1 wherein the polymer is a compound selected from the group consisting of polyvinylpyrrolidone, polyethylene glycol, dextran, polyoxyethylene-polyoxypropylene copolymer, polyvinyl alcohol, and mixtures thereof.

6. The method of claim 1 further comprising oxidizing the aldehyde with an oxidizing agent prior to reacting the reaction mixture.

7. The method of claim 1 wherein the conjugates are formed through Schiff base intermediates.

8. The method of claim 1 wherein the macromolecular conjugate is selected from the group consisting of a carbohydrate-protein conjugate, protein-protein conjugate, protein-peptide conjugate, and protein-label conjugate.

9. The method of claim 8 wherein the macromolecular conjugate is a carbohydrate-protein conjugate and the carbohydrate is derived from a bacterium selected from the group consisting of Micrococcaceae, Streptococcaceae, Lactobacillaceae, Propionibacteriaceae, Bacillaceae, Neisseriaceae, Brucellaceae, Pseudomonadaceae, Spirillaceae, Actinomycetales, Spirochetales, Mycoplasmataceae, Rickettsiaceae, Chlamydiaceae, Bartonellaceae, fungal groups, Phycomycetes, protozal groups, and virally associated lipopolysaccharides, polysaccharides and oligosaccharides.

10. The method of claim 8 wherein the protein is selected from the group consisting of tetanus toxoid, diphtheria toxoid, *Neisseria meningitidis* outer membrane protein, nontoxic cross-reacting mutant of diphtheria toxin, a protein derived from bacteria, exotoxins and recombinant derivatives thereof, and a synthetic protein containing lysine residues.

12. The method of claim 1 wherein the mixture is reacted at a pH near the pI of the resulting conjugate.

13. A method for preparing a purified macromolecular conjugate comprising reacting a reaction mixture containing a macromolecular conjugate with a polymer for a sufficient time to form conjugate particles and separating the particles from the reaction mixture.

14. The method of claim 13 further comprising solubilizing the separated particles.

15. The method of claim 13 wherein the polymer is a compound selected from the group consisting of carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers and block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched and cyclo-polymers, and polyaldehydes, and mixtures thereof.

16. A method for preparing a purified macromolecular conjugate comprising reacting a reaction mixture containing molecules to be conjugated and a conjugating agent with a polymer for a sufficient time to form conjugate particles and separating the particles from the reaction mixture.

17. The method of claim 16 further comprising solubilizing the separated particles.

18. The method of claim 16 wherein the polymer is a compound selected from the group consisting of carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers and block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched and cyclo-polymers, and polyaldehydes, and mixtures thereof.

19. A macromolecular conjugate prepared by reacting a reaction mixture comprising a molecule having a reactive amine group, a molecule having a reactive aldehyde group, and a polymer for a sufficient amount of time to form conjugate particles and separating the particles from the mixture, wherein the particles are comprised of the macromolecular conjugate.

20. The conjugate of claim 19 further comprising solubilizing the separated particles.

21. The conjugate of claim 19 wherein the polymer is a compound selected from the group consisting of carbohydrate-based polymers, polyaliphatic alcohols, poly(vinyl) polymers, polyacrylic acids, polyorganic acids, polyamino acids, co-polymers and block co-polymers, tert-polymers, polyethers, naturally occurring polymers, polyimids, surfactants, polyesters, branched and cyclo-polymers, and polyaldehydes, and mixtures thereof.

22. The conjugate of claim 19 further comprising oxidizing the aldehyde with an oxidizing agent prior to reacting the reaction mixture.

23. The conjugate of claim 19 wherein the conjugate is formed through a Schiff base intermediate.

24. A macromolecular conjugate microparticle comprising a non-resilient outer surface encapsulating an inner matrix, wherein both the outer surface and the inner matrix are comprised of homogeneously distributed and intertwined macromolecule conjugate and polymer, wherein the inner matrix is water soluble and the solubilized inner matrix diffuses through the outer surface.

25. The microparticle of claim 24 having a spherical shape and a diameter between approximately 1 and 10 μm.

26. A method for immunizing a human or animal comprising administering to the human or animal a sufficient amount of a purified conjugate prepared by a method comprising reacting a reaction mixture comprising a molecule having a reactive amine group, a molecule having a reactive aldehyde group, and a polymer for a sufficient amount of time to form conjugate particles and separating the particles from the reaction mixture.

27. The method of claim 26 further comprising solubilizing the separated particles prior to administration.